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Incompatibility properties of the narrow-host-range lactococcal plasmid pCI305

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The cryptic plasmid pCI305 from *Lactococcus lactis* subsp. *lactis* UC317 was screened for incompatibility with a range of cloning vectors and was found to be incompatible with the theta-replicating plasmids pAM401 and pIL253. In contrast, lactococcal vectors containing the replication functions of the rolling circle replicating plasmids pSH71 and pWV01 were compatible with pCI305. A number of native lactococcal plasmids were also screened for both incompatibility and DNA sequence homology to the pCI305 replicon. A plasmid with high sequence identity was identified which was compatible with pCI305.

Introduction

Lactococcus lactis strains are widely used in the manufacture of a range of fermented food products and consequently are of considerable industrial and economic importance. Many of the key technological traits of these bacteria, e.g. proteinase activity (Prt), lactose metabolism (Lac) and bacteriophage resistance mechanisms such as abortive infection (Abi) and adsorption inhibition (Ads), are plasmid-encoded and have been studied extensively (de Vos, 1986). However, there is little information available concerning the intrinsic properties of these plasmids, e.g. replication mechanisms, stability and incompatibility functions. Knowledge of these characteristics is essential for the construction of strains of lactococci with improved technical traits and enhanced reliability.

The replication characteristics of a number of small lactococcal plasmids, used to construct a range of cloning vectors for the genetic manipulation of lactic acid bacteria, have been described. These molecules, which include pSH71 (de Vos, 1986, 1987) and pWV01 (Leenhouts *et al.*, 1991), replicate by a rolling circle replication (RCR) mechanism (Gruss & Ehrlich, 1989) which may explain their high structural and segregational instability (Bron & Luxen, 1985; Bron *et al.*, 1988; Ehrlich *et al.*, 1986). Hayes *et al.* (1990) identified a small cryptic plasmid, pCI305, from *L. lactis* subsp. *lactis* UC317 which, unlike the replicons described above, is extremely stable (both structurally and segregationally) and has a narrow host-range. The inability to detect single-stranded DNA (an intermediate of RCR) and the lack of DNA sequence homology to the highly conserved replication regions of RC plasmids (Hayes *et al.*, 1991) would suggest that pCI305 probably uses a theta-mode of replication. In addition, several features of the pCI305 minimal replicon are to be found in many of the theta-replicating plasmids from Gram-negative bacteria, e.g. pSC101 (Manen & Caro, 1991), RSF1010 (Persson & Nordstrom, 1986) and R1162 (Lin *et al.*, 1987).

An important intrinsic characteristic of plasmids which needs to be considered when devising strain improvement strategies relates to incompatibility. Bacterial plasmids that share either replication control or partitioning functions and which compete for stable inheritance are termed incompatible and are placed in the same incompatibility group (Novick *et al.*, 1976). A study of incompatibility groups amongst native lactococcal plasmids should provide considerable information concerning the evolutionary relatedness of these molecules and could lead to the construction of different classes of stable cloning vectors which can avoid incompatibility reactions when used in various lactococcal hosts. Thus far there have been no comprehensive reports concerning plasmid incompatibility in lactic acid bacteria. However, incompatibility has been shown to result in plasmid curing in *L. lactis* subsp. *cremoris* Wg2 (van der Lelie *et al.*, 1988), *Lactobacillus pentosus* (Posno *et al.*, 1991), *Lb. acidophilus* (Muriana & Klaenhammer, 1987) and *Lb. plantarum* (Bringel *et al.*, 1989).

In this paper the incompatibility properties of the

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Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Characteristics	Reference or source
L. lactis subsp. *lactis*		
MG1363	Lac$^-$ Prt$^-$ Sm^r plasmid-free derivative of *L. lactis* NCDO 712	Gasson (1983)
MG1614	Lac$^-$ Prt$^-$ Sm^r Rif^r derivative of MG1363	Gasson (1983)
FH052	MG1363Sm^r harbouring pCI305	Hayes *et al.* (1990)
FH136	MG1363Sm^r harbouring pCI350	Hayes *et al.* (1990)
MG1299	MG1363Sm^r harbouring pLP712	Gasson (1983)
UC505	MG1363Sm^r harbouring pCI528	Costello (1988)
AC002	MG1363Sm^r harbouring pCI829	Coffey *et al.* (1989)
AC004	MG1363Sm^r harbouring pCI842	Coffey *et al.* (1989)
MM106	LM2306 harbouring pC1726	Murphy (1988)
AB002	MG1614 harbouring pC1750	Baumgartner *et al.* (1986)
DPC3260	MG1614 harbouring pNP40	Harrington & Hill (1991)
Plasmids		
pC1305	8·7 kb cryptic plasmid from *L. lactis* subsp. *lactis* UC317	Hayes *et al.* (1990)
pC1350	pCI305 cloned into replication probe vector pCI341, Cm^r (11·8 kb)	Hayes *et al.* (1990)
pC1369	2·6 kb replicon of pCI305 subcloned into pCI341, Cm^r (5·7 kb)	Hayes *et al.* (1990)
pCI374	1·8 kb replicon of pCI305 subcloned into pCI341, Cm^r (4·9 kb)	Hayes *et al.* (1990)
pAM401	Cm^r Tc^r, pIP501 replicon (10·4 kb)	Wirth *et al.* (1986)
pIL253	Em^r, pAMβ1 replicon (4·8 kb)	Simon & Chopin (1988)
pGKV210	Em^r, pWV01 replicon (4·4 kb)	van der Vossen *et al.* (1985)
pGKV110	Em^r, pWV01 replicon (4·4 kb)	van der Vossen *et al.* (1985)
pNZ521	Cm^r Km^r, pSH71 replicon (10·5 kb)	de Vos *et al.* (1989)
pCI3601	Cm^r Km^r, pSH71 replicon (10·5 kb)	Law *et al.* (1992)
pNP40	Abi Nis^r plasmid (64 kb) of *L. lactis* subsp. *lactis* biovar. *diacetylactis* DRC3	McKay & Baldwin (1984)
pCI528	Ads plasmid (46 kb) of *L. lactis* subsp. *cremoris* UC503	Costello (1988)
pCI829	Abi plasmid (44 kb) of *L. lactis* subsp. *lactis* UC811	Coffey *et al.* (1989)
pCI842	Lac$^+$ plasmid (61 kb) of *L. lactis* subsp. *lactis* UC811	Coffey *et al.* (1989)
pCI750	Abi plasmid (65 kb) of *L. lactis* subsp. *cremoris* UC653	Baumgartner *et al.* (1986)
pCI726	Lac$^+$ plasmid (40 kb) of *L. lactis* subsp. *cremoris* UC653	Baumgartner *et al.* (1986)
pLP712	Lac$^+$ Prt$^+$ plasmid (51 kb) of *L. lactis* subsp. *lactis* 712	Gasson (1983)

narrow-host-range cryptic lactococcal plasmid pCI305 are described. This includes a study of the interactions of pCI305 with several native lactococcal plasmids, known to encode a variety of industrially important traits, and also with several frequently used cloning vectors.

Methods

Bacterial strains, media and reagents. The bacterial strains used in this study are described in Table 1. All lactococcal strains were routinely subcultured at 30 °C in M17 medium (Terzaghi & Sandine, 1975) supplemented with 0·5% glucose (GM17). Solid media contained 1·5% (w/v) agar (Oxoid no. 3). Erythromycin and chloramphenicol were used when required at a final concentration of 5 μg ml^{-1}.

Isolation of plasmid DNA and transformation procedures. Large-scale and rapid isolation of plasmid DNA from lactococci was achieved using the method described by Anderson & McKay (1983). Purification of plasmid DNA by caesium chloride/ethidium bromide density gradients was performed as described by Maniatis *et al.* (1982). Lactococcal strains were transformed by electroporation using the procedure of Holo & Nes (1989).

Southern hybridization. DNA (either unrestricted or linearized) was electrophoresed on 0·7% horizontal or vertical agarose gels, transferred to Hybond N$^+$ nylon filters by the method of Southern (1975) and hybridized with a DNA probe labelled using the Enhanced Chemiluminescence (ECL) gene detection system (Amersham). All protocols were performed as suggested by the suppliers. The DNA fragment used as the probe was recovered from an agarose gel using the Geneclean kit (Bio101).

Incompatibility studies. L. lactis cultures each containing a specific resident plasmid were transformed by electroporation with the test plasmid DNA and plated on a medium selective for the incoming plasmid. Transformants were grown in GM17 broth in the absence of antibiotic selection for 4 h (approximately four generations) and screened for the retention of the resident plasmid by agarose gel

electrophoresis. Those transformants which were found to contain both plasmids were subcultured for 100 generations without selective antibiotic pressure. During this time the cultures were maintained in the exponential phase of growth by extensive dilution at 10 h intervals. After 50 and 100 generations, appropriate dilutions were plated on nonselective medium and subsequently 100 colonies were replica-plated onto selective plates. Up to 10 single colonies of each phenotype (antibiotic resistant/sensitive) were screened for the presence of the original resident plasmid, the transforming plasmid or both by agarose gel electrophoresis. The percentage of the population in which loss of a specific plasmid occurred was then determined.

Results and Discussion

Screening of vector plasmids for incompatibility with pCI305

Several cloning vectors commonly used in the genetic manipulation of lactococci were screened for incompatibility with pCI305. In these experiments the cryptic plasmid, pCI305, was the resident replicon and selection was for the incoming plasmid only. The results are described in Table 2. In all cases where loss of antibiotic resistance occurred, this was due to the elimination of the corresponding plasmid based on agarose gel electrophoresis analysis of plasmid content. Two types of incompatibility were detected. Incompatibility was first observed when chloramphenicol-resistant derivatives of pCI305 were used as the incoming plasmid. Examination of the plasmid content of antibiotic-resistant transformants following only three to four generations of growth in broth in the absence of selective pressure revealed that both the incoming and resident plasmids were present. However, when this heteroplasmid strain was subcultured for 100 generations under nonselective conditions homoplasmid segregants were isolated (see Table 2). This type of incompatibility between pCI305 and its chloramphenicol-resistant derivatives was to be expected since all of these molecules have identical replication functions. It is likely that in this case plasmid incompatibility occurred due to the random selection, for replication and partitioning events, of individual copies of either molecule within the plasmid pool. According to Novick (1987), in such cases homoplasmid segregants will appear at a constant rate during growth of the heteroplasmid strain. When the same experiment was repeated with the modification of growing the antibiotic-resistant transformants, initially for four generations, in the presence of antibiotic selective for the incoming plasmid, a distortion of the average copy pool was observed (data not shown). This distortion was reflected in the ratio of homoplasmid segregants containing one or the other plasmid following 100 generations of growth in the absence of selective pressure (e.g. 81% of the population contained pCI369 in contrast to 37% when hosts were grown continuously in the absence of selective pressure).

Table 2. *Incompatibility between pCI305 and a range of vector plasmids*

Incoming plasmid†	Percentage of population after 100 generations with: pCI305 only	Incoming plasmid only	Both plasmids	Incompatibility with pCI305‡	Hybridization to pCI305§
pCI350	57	43	0	INC	+
pCI369	63	37	0	INC	+
pCI374	62	38	0	INC	+
pAM401	100	0	0	INC	−
pIL253	100	0	0	INC	−
pGKV210	0	0	100	COM	−
pGKV110	0	0	100	COM	−
pNZ521	11	0	89	COM*	−
pCI3601	33	0	67	COM*	−

†The host strain in each case was *L. lactis* subsp. *lactis* FH052 (MG1363 containing pCI305).

‡INC, 100% loss of one plasmid; COM, 100% coexistence of both plasmids; COM*, coexistence of both plasmids in > 60% of the population after 100 generations.

§The probe used was a 1·8 kb *Xba*I–*Pst*I fragment of pCI374 containing the pCI305 minimal replicon.

Vectorial incompatibility (i.e. where one plasmid is lost exclusively; Novick, 1987) between pCI305 and the theta-replicating plasmids pAM401 and pIL253 was the second type of interaction observed (Table 2). The plasmid content of these antibiotic-resistant transformants was examined following only four generations of growth under non-selective conditions and in both cases only the resident plasmid, pCI305, was present. Therefore the incompatibility between these two plasmids (pAM401 and pIL253) and pCI305 results in rapid loss of the incoming plasmid in the absence of selective pressure. It has frequently been reported that when the incoming and resident plasmids are incompatible, transformation of the host occurs at a reduced efficiency (Bird & Pittard, 1982; van der Lelie *et al.*, 1988; Warren & Sherratt, 1978). However, in the experiments described in this study the nature of the resident or incoming plasmid did not appear to alter transformation frequencies.

Both pAM401 and pIL253 use a theta-mode of replication (Brantl *et al.*, 1990; Bruand *et al.*, 1991) and may therefore be incompatible with pCI305 due to competition for a common factor involved in replication or partitioning. pIP501 and pAMβ1 (the sources of the pAM401 and pIL253 replicons, respectively) belong to the same incompatibility group on the basis that they are incompatible with each other and they share extensive similarities in the DNA sequence and organization of their replication regions (Brantl *et al.*, 1990). However, no homology could be detected in hybridization experi-

Table 3. *Screening of native lactococcal plasmids for incompatibility with the pCI305 replicon*

Resident plasmid†	Relevant phenotype	Incompatibility with pCI350‡	Hybridization to pCI305§
pNP40	Abi Nis^r	INC	−
pCI528	Ads	COM	+
pCI829	Abi	COM	+*
pCI842	Lac	INC	+*
pCI750	Abi	COM	−
pCI726	Lac	COM*	+
pLP712	Lac Prt	COM	−

† The incoming plasmid used was pCI350 (a derivative of pCI305 harbouring a chloramphenicol-resistance determinant). The host strain was *L. lactis* subsp. *lactis* MG1363 for pCI528, pCI829, pCI842, pLP712; *L lactis* subsp. *lactis* MG1614 for pNP40 and pCI750; *L. lactis* subsp. *lactis* LM2306 for pCI726.

‡ INC, 100% loss of the incoming plasmid, pCI350; COM, 100% coexistence of both plasmids; COM*, coexistence of both plasmids in 51% of the population after 100 generations.

§ The probe used was a 1·8 kb *Xba*I–*Pst*I fragment of pCI374 containing the pCI305 minimal replication region. +* represents weak hybridization.

ments between these plasmids and the minimal replication region of pCI305 (Table 2). The reasons for the strong incompatibility between pCI305 and replicons derived from pAMβ1 and pIP501 are unknown. Since there is no DNA sequence homology between their replication regions it is possible that a segment of pCI305 outside of the minimal replicon is involved. Such a sequence could possibly encode a stability/maintenance function or a factor inhibitory to pAM401 and pIL253 replication or maintenance, resulting in complete loss of these plasmids in the presence of pCI305. Another possibility is that there is competition for a host factor between the plasmids and since pCI305 is a lactococcal plasmid it may have a distinct advantage in contrast to the pAMβ1 replicon of *Enterococcus faecalis* and pIP501 from *Streptococcus agalactiae*.

Cloning vectors based on the cryptic RC plasmids pSH71 (de Vos, 1987) and pWV01 (Leenhouts *et al.*, 1991) were found to be compatible with pCI305 (Table 2) and as previously reported (Hayes *et al.*, 1991), no DNA sequence homology to the minimal replicon of pCI305 was detected. It will thus be possible to examine the effect of specific sequences from the pCI305 minimal replicon on pCI305 replication when these sequences are provided *in trans*.

Incompatibility between native lactococcal plasmids and the pCI305 replicon

Numerous plasmids of enteric bacteria and *Staphylococcus* are very well characterized (in comparison to those of lactic acid bacteria) and many have been classified into incompatibility groups (Couturier *et al.*, 1988; Iordanescu *et al.*, 1978; Novick, 1989; Udo & Grubb, 1991). Since plasmid incompatibility can occur due to competition for a common factor involved in plasmid replication or partitioning, a correlation has frequently been made between DNA sequence homology and incompatibility (e.g. the IncI group of enteric plasmids; Falkow *et al.*, 1974). If such observations could be made amongst the lactococcal plasmids it would greatly facilitate the classification of these replicons. Such grouping of plasmids would provide considerable information concerning the evolutionary relatedness of these molecules within *L. lactis* subsp. *lactis* and *cremoris*.

An experiment by Hayes *et al.* (1991) in which the plasmid complements of eight multi-plasmid *Lactococcus* strains were screened for DNA homology to the pCI305 minimal replication region illustrated the widespread distribution of the 'pCI305-type' replicon. In Table 3 results are presented describing an experiment in which several native lactococcal plasmids with known functions were screened for incompatibility with pCI305. In all cases pCI350, a derivative of pCI305 harbouring a chloramphenicol-resistance determinant, was used as the incoming plasmid. The native plasmids were also screened for DNA sequence homology to the pCI305 replicon in order to identify those plasmids which were incompatible due to shared replication functions. The probe used was a 1·8 kb *Xba*I–*Pst*I pCI374 fragment containing the minimal replicon of pCI305. Sequence analysis of this fragment indicated the absence of any of the known IS elements or transposons which are ubiquitous in lactococcal DNA. The results indicated that there was not necessarily a correlation between DNA sequence homology in the replication regions and incompatibility. This can be seen with pCI528, which exhibited extensive homology to the minimal replicon of pCI305 yet these two plasmids were compatible. In addition, plasmids were identified which were incompatible with pCI305 but this was not due to common replication functions. For example, the phage-resistance plasmid, pNP40, was incompatible with pCI350 but did not hybridize to the pCI305 probe (Table 3).

Comparison of the DNA sequence of the pCI528 replicon (M. Lucey, unpublished results) with that of pCI305 revealed greater than 70% homology. It was also observed in *in vivo* experiments that the replication protein of pCI528 could not act *in trans* on the replication origin (*repA*) of pCI305 (unpublished data). Thus it is likely that the sequence differences within the replication origin and the gene encoding the replication protein enable these plasmids to coexist and be stably maintained. Analysis of differences such as those between pCI305 and pCI528 should assist in elucidating the

factors that determine plasmid incompatibility/compatibility and replication control.

Further analysis of these various interactions is required to understand how pCI305 is incompatible with the theta-replicating plasmids pIL253 and pAM401 and compatible with plasmids of high sequence identity, e.g. pCI528. This may result in the identification of functions outside of the minimal replicon which are responsible for the stable maintenance of pCI305. In addition, the identification of the specific nucleotide base differences between pCI528 and pCI305 that enable independent replication of these plasmids should contribute to our understanding of the replication mechanisms of pCI305 and related plasmids.

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